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Biomolecular evaluation of three contrasting rice cultivars (*Oryza sativa* L.) in salt stress response at seedling stage

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Abstract

Salt contamination of soils due to climate change faces a severe environmental issue that affects crop production today. However, the response mechanism in plants to salt stress is not fully understood. The present study investigated molecular and biochemical changes under salt stress in rice seedlings of three rice cultivars, *i.e.*, AGPPS114 (salt-tolerant), OM6967 (moderately tolerance), VD20 (salt-sensitive). Increasing salt concentration leads to a reduction in shoot/root length but different levels among the cultivars. In contrast, reactive oxygen species (ROS) accumulation and lipid peroxidation increased progressively with increasing salt concentration and time course treatment. However, at 250 µM of NaCl, these parameters were more adversely affected in VD20 than AGPPS114 and OM6967. Using ICP showed that Na^+ accumulation in rice root increased gradually with increasing NaCl concentrations in all cultivars under salt treatment but was low in salt-sensitive cultivar VD20 compared to other cultivars. Antioxidant enzyme activity analysis indicated catalase (CAT) and superoxide dismutase (SOD) were induced during salt treatment in all cultivars. The results also showed greater proline and glycine betaine accumulation in the AGPPS114 than OM6976 and VD20. qPCR indicated a significant difference in transcript levels of the Na^+-transporter gene *OsSOS1*, *OsNHX1* and *OsHKT1s* in AGPPS114 and OM6967 cultivars compared to VD20 cultivar. In summary, the active regulation of genes related to Na^+ transport at the transcription level and with high glycine betaine and proline accumulation levels may be involved in salt tolerance mechanisms and thus might be useful for selecting tolerant plants.

Keywords

ROS, quantitative real-time PCR, rice (*Oryza sativa* L.), isozyme, salt tolerance, Na+-transporter gene

Introduction

Soil salinization is a serious constraint on both agricultural production and crop yield that adversely affects food security, particularly in arid and semi-arid regions (1). Globally, it is estimated that nearly 380 million ha of the total rice land area is affected by salinity (2). Due to global climate change, Vietnam is one of the countries most affected by salinity. The Red River Delta and Mekong Delta, two main rice cultivated areas of Vietnam, are flooded in saltwater. In 2017, salinity severely harmed 215,445 ha of rice

land in the Mekong River Delta, resulting in direct economic losses of VND 7,517 billion (about USD 337 million) (3). Land salinization has become an important factor limiting the sustainable development of agriculture in the Mekong River Delta.

Rice is a major crop grown on 156 million ha of land worldwide (yielding 650 million tons of grain), accounting for 60 % of food consumption in Southeast Asia and roughly 35 % in East and South Asia (4, 5). Rice is a salt-sensitive crop; however, there are various salt tolerance genetic variation levels in the cultivated rice gene pool at different growth stages (6-8). A greater understanding of this genetic diversity of rice's response to salinity stress may benefit for salt tolerance improvement in rice. Many traditional rice cultivars and landraces are more resistant to salinity stress than many elite cultivars and tolerant genotypes are evaluated as good sources of tolerance traits (9).

At the physiological level, the wide range of effects of salt stress emphasizes the importance of shielding plants from damage caused by reactive oxygen species (ROS) that unavoidably increase due to increased ion uptake and water deficit impair photosynthesis (10-12). Also related are the protective roles played by the aggregation of metabolites, which appear to serve multiple functions such as preventing radical formation, adding to redox control, acting as low molecular weight chaperones and working as compatible solutes by decreasing the osmotic potential (13-16). Studies on the components engaged in relative resistance have recognized a few proteins that decide the endpoints of physiological responses (16). Work on transgenic plants supports the idea that changed gene expression can improve tolerance (17, 18). Furthermore, elements of abiotic stress signal detection and transduction pathways, as well as transcription activators, have recently been identified (19-22). These elements, for example, a calcium sensor's impact on a protein kinase that influences a Na^+/H^+ antiporter, initiate and regulate the expression of downstream metabolic events (23-25).

The cultivars, growth stages, and organ types are significant factors to consider when selecting rice against salinity stress. Many studies have demonstrated that salt stress affects rice growth at all stages of its life cycle, from germination to maturity, although the seedling stage is the most vulnerable (8, 26-28). To better understand the physiological and molecular responses to salt stress, this study investigated the salinity stress response in the root seedlings of three widely used rice cultivars (VD20 - salt-sensitive genotype, OM6976 - moderately salt-tolerance and AGPPS114 - high salt-tolerance). The biochemical and molecular profiling could provide an initial functional insight into the metabolic pathways that lead to tolerance acquisition.

Materials and Methods

Plant materials and treatment

The 12 rice cultivars (*Oryza sativa* var. indica) supplied by Loc Troi Group (Vietnam) and Southern Seed Corporation (Vietnam) were evaluated for salt tolerance using the IRRI standard evaluation scoring system based on visual symptoms of salt injury at the seedling stage (Supplementary Table 1) (29). Three cultivars, including VD20 (salt-sensitive), OM6976 (moderately salt tolerance) and AGPPS114 (high salt tolerance), were selected for this study. VD20 is a short-term fragrant rice cultivar with a growing cycle of 100-115 days in the winter-spring crop season. The tree height is from 105-115 cm. VD20 cultivar is grown mainly in the Mekong River Delta (Vietnam). OM 6976 rice cultivar has good tillering, tree height from 95-100 cm, very hard, big bunches of cotton, thick seeded seeds, can be grown in many types of soil due to the characteristics of resistance to alum and salinity. OM6976 is cultivated in 2 seasons, the winter-spring crop season with a growing cycle of 125-130 days and the summer-autumn crop season with a growing cycle of 105 - 110 days (30). AGPPS114 is a short-term fragrant rice cultivar with a growing cycle of 93-97 days that can cultivate 3 crops per year. The tree height is from 95-100 cm (31).

The cultivar seeds were surface sterilized for 15 min. with 2.5 % (v/v) sodium hypochlorite, then rinsed twice with deionized water. Two grams of surface sterilized seeds were incubated for 3 days at 37 °C in darkness in 9 cm Petri dishes which contained 20 ml deionized water. The 10 germinated seeds were cultivated for 3 days at 28 °C in the dark on 9 cm Petri plates on filter paper discs moistened with 10 ml deionized water. The root seedlings were exposed to salinity stress with varying concentrations (0 to 500 mM) of NaCl in the same Petri dish after reaching 2-3 cm in length. After 3 days of incubation in the dark at 28 °C, the shoot and root length was measured. The mean of shoot and root length was obtained from 10 individual seedlings from at least 3 separate experiments. ROS production, proline content, in-gel isozyme activity, semi-quantitative PCR (RT-PCR) and real-time PCR (qPCR) analysis were all determined on the roots of NaCl-treated rice seedlings.

Determination of Na+ accumulation and Na+ localization in rice roots

Six-day-old rice seedlings of the cultivars were exposed to salt stress for three days to determine Na^+ concentration. The roots of treated-rice seedlings were harvested, dried and washed in pure nitric acid. 100 mg dry weight of root samples were placed in a Teflon vessel for 1 hr. In total, 7 ml of 10 % (v/v) ultra-pure hydrochloric acid (Sigma-Aldrich, USA) and 21 ml of 10 % (v/v) ultra-pure nitric acid (Sigma-Aldrich, USA) were added to the container, which was then placed in a Teflon bomb and microwaved for 30 min. at 600 W in a microwave digester (Ethos Easy, Milestone, Italy). The content of Na^+ in rice roots of the cultivars was measured in 3 different biological replicates of root samples by Spectroblue ICP-OES (Inductively coupled plasma - optical emission spectrometry, Ametek, Germany).

Histochemical localization to detect Na^+ localization in rice roots was conducted as per the procedure (32). The root tissues of rice seedlings were incubated in chilled medium A consisted 10 mM CoroNa Green indicator

(Molecular Probes, USA) and 0.02 % non-ionic detergent Pluromic F-127 for one hour. The rice roots were then cleaned twice in deionized water before being examined using an Olympus BX53 fluorescent microscope (Olympus, Tokyo, Japan) at wavelength 516 nm and photographed with a camera Olympus DP73 (Olympus, Tokyo, Japan).

Reactive oxygen species (ROS) and lipid peroxidation detection

Hydrogen peroxide (H_2O_2) and superoxide anion radicals ($O_2^{\cdot-}$) and accumulation in rice roots under salt stress were indicated by staining with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) respectively (33). Rice roots were treated with NaCl solutions for 24 hrs. For H_2O_2 staining, roots were steeped in a 10 mM MES buffer, pH 3.8 consisted of 1 mg/ml DAB for 8 hrs in the dark. In order to detect $O_2^{\cdot-}$, rice roots were incubated with 0.5 mg/ml NBT (Sigma-Aldrich) produced in 10 mM NaN_3 in 10 mM potassium phosphate buffer, pH 7.8. An Olympus SXZ16 stereomicroscope (Olympus, Tokyo, Japan) was used to examine the stained rice roots.

Schiff's reagent was used to detect lipid peroxidation in rice roots during salt stress (34, 35). To identify aldehydes resulting from lipid peroxides, the roots of salt-treated seedlings were treated with Schiff's reagent for 60 min. in the dark. To keep the staining color, the roots were immersed in a sulfite solution (0.5 % [w/v $K_2S_2O_5$ produced in 0.05 M HCl]) for ten min.

Proline and glycinebetaine level determination

Quantification of free proline content was measured as per standard methodology (36). Root samples (0.5 g) were ground in 1 ml of 3 % (w/v) sulfosalicylic acid and homogenate centrifuged at 13000 rpm for 10 min. at 4 °C. The addition of 0.2 ml of acid ninhydrin (0.31 g ninhydrin prepared in 7.5 ml acetic acid and 5 ml of 6 M phosphoric acid), 0.2 ml of glacial acetic acid and 0.1 ml of 3 % sulfosalicylic, the resulting rice mixture was boiled for one hour. The absorbance at 520 nm of the fraction with toluene aspirated from the liquid phase was measured after the rice mixture was extracted with 1ml toluene. The calibration curve was used to determine proline content and expressed as mmol proline/g fresh weight.

Glycinebetaine was analyzed following the standard method (37). Rice roots were collected and dried at 80 °C for four days. Dried, finely powdered root seedlings (0.5 g) were incubated for 24 hrs at 25 °C in 20 ml of deionized water. The extracts were diluted 1:1 with 2 N H_2SO_4. Aliquots of 0.5 ml were cooled in ice water for one hr, and then 200 µl of cold KI-I_2 reagent (15.7 g of Iodine and 20 g of KI in 100 ml deionized water) was added. The mixture was kept at 4 °C for 16 hrs before being centrifuged at 10000 rpm for 15 min at 4 °C. The supernatant was collected and then added with 5 ml of 1,2-dichloroethane. The absorbance was measured at 365 nm after 2-2.5 hrs.

Antioxidant enzyme assays

The seedlings of VD20, OM6976 and AGPPS114 cultivar were treated with 100 mM, 150 mM and 250 mM, respectively, of NaCl concentration (the half maximum inhibitory concentration – IC_{50}). After salt treatments, protein content on rice root was extracted following the standard method (38). Approximately 1 cm long root tips of 30 rice seedlings were excised and ground in extract buffer containing 250 mM sucrose, 10 mM Na_3VO_4, 10 mM NaF, 1 mM sodium-tartrate, 10 percent v/v glycerol, and 50 mM $Na_2S_2O_5$ supplemented with 1 mM phenylmethanesulfonyl fluoride prepared in 50 mM Tris-HCl pH 7.4, with the use of a grinder in the tube. The homogenates were centrifuged at 13000 rpm for 10 min at 26 °C, and the supernatants were saved. The BioRad Dc Protein Assay (Bio-Rad, Hercules, CA, USA) was used to assess protein concentration with OD at 750 nm.

Activities of superoxide dismutase (SOD) were determined by measuring its capacity to inhibit the photochemical reduction of NBT (39). A 3 ml aliquot of reaction mixture contained 30 µl of enzyme extract, 50 mM phosphate buffer (pH 7.8), 56 mM NBT, 1.17 mM riboflavin, 10 mM methionine. The reaction was performed by exposing the mixture to white light for 15 min. at room temperature. The absorbance of the reaction mixture was detected with OD at 560 nm. Analysis of catalase (CAT) activity was assayed by measuring the initial rate of disappearance of hydrogen peroxide (H_2O_2) as described (40). The reaction mixture in a total volume of 3 ml contained 0.1-0.2 ml of enzyme extract, 0.4 ml of 200 mM H_2O_2 and 1 ml of 100 mM phosphate buffer (pH 7.0). The reduction in absorbance was recorded with OD at 240 nm for 30 seconds.

Protein solution (10 µg) was separated on native polyacrylamide gel electrophoresis (Native PAGE) using a 4.5 % stacking gel combined with 10 % polyacrylamide separating gel at constant voltage (100 V) for 2.5 hr at 25 °C. In-gel catalase and SOD activity were analyzed following the method described (41). Briefly, the gels were initially incubated in 0.3% H_2O_2 for 20 min. to detect catalase activity. The gels were then gently rinsed in deionized water for 1 min., before being developed in a reaction mixture containing 1% (w/v) ferricyanide and 1% ferric chloride solution (w/v) for 15 min. To detect SOD isoenzymes, the gels were washed in deionized water and incubated in the dark for 30 min. at room temperature in a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM NBT and 0.3% *N*,*N*,*N''*,*N''*-tetramethylethylenediamine. The gels were then rinsed with deionized water and exposed on a lightbox for 10 min. at room temperature and the colorless bands of SOD activity in a purple-stained gel were visible. Coomassie blue staining was used to confirm the results of the protein assay.

Gene expression analysis by reverse transcriptase - PCR (RT-PCR)

Root seedlings (100 mg) of VD20, OM6976 and AGPPS114 rice cultivar treated with 100 mM, 150 mM and 250 mM NaCl solution respectively, for different time courses were collected for total RNA extraction, using GeneJET RNA Purification Kit (Thermo Scientific, USA) with several modifications. The total RNA sample concentrations were measured using Biophotometer Plus (Eppendorf, USA) and µCuvette™ G1.0 (Eppendorf, USA). The purity and concentration of RNA solutions were measured using ratios $OD_{260/280}$ and $OD_{260/230}$. High quality total RNA samples ($OD_{260/280}$ > 2, $OD_{260/230}$ > 2),

containing more than 2 µg/µl of total RNA, were used for RT -PCR reactions. The thermal cycle of PCR was initial denaturation at 94°C for 2 min., followed by 30 cycles of amplification (94 °C for 30 seconds, primer-specific annealing temperature for 1 min. and 72°C for 2 min.) and final elongation at 72 °C for 10 min. Rice *α-tubulin* gene (*Os03g0726100*) was used as a reference gene. Amplicons were detected on an agarose gel (1.5%). Three independent biological replicates were conducted for each gene expression measurement. Oligonucleotide primers for RT-PCR are listed in Table 1.

Table 1. The forward and reverse primer sequences applied in this study

source	Forward Primer Sequence	Reverse Primer Sequence
Semi-quantitative RT-PCR primers		
OsSOS1 (Os12g0641100)	5' GGACGCAAACAGACGCCAAAGT 3'	5' TTTTTCCGCTGTCTCCTGCTCA 3'
OsNHX1 (Os07g0666900)	5' TTGGAACGCTGGATGTAGGA 3'	5' GGGCAACCTCACGGTCAGTA 3'
OsHKT1;4 (Os04g0607600)	5' AGTGACAGGTTATTTCGTAGC 3'	5' AGTTCTTTCTGAAGGGTTGC 3'
OsHKT1;5 (Os01g0307500)	5' AAGTGGTTAGGGACATTACA 3'	5' AACCTCAATAGTGGCGATA 3'
α-tubulin (Os03g0726100)	5' TCGCAGCATCAACCCAATC 3'	5' GCAACCAGTCCTCACCTCAT 3'
Quantitative real-time PCR primers		
OsSOS1 (Os12g0641100)	5' ATTTGGTCTCGAGTCATCAG 3'	5' GATATCAATGACGCACTCCT 3'
OsNHX1 (Os07g0666900)*	5' GCTAGATTTGAGCGGCATTC 3'	5' CACTGGCAAACTCCCATTTT 3'
OsHKT1;4 (Os04g0607600)*	5' CATCTGCATCACCGAGAGAA 3'	5' CTCCCTACGAAACCAGTCCA 3'
OsHKT1;5 (Os01g0307500)*	5' CCCATCAACTACAGCGTCCT 3'	5' AACTTCTTGAGCCTGCCGTA 3'
α-tubulin (s03g0726100)	5' AGGTTATCTCATCCCTGACC 3'	5' GTAGGACGAAAGCATGAAGT 3'

Quantitative real-time PCR analysis (qPCR)

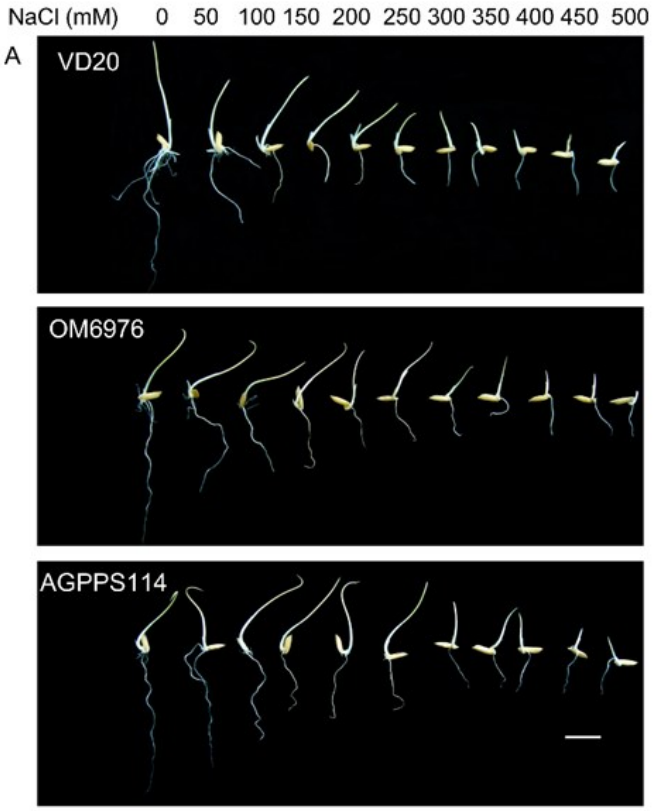
qRT-PCR was performed to measure the expression levels of the genes of interest using iQ™ SYBR® Green Supermix (Bio-RAD, USA) on the StepOnePlus Real-Time PCR (Applied Biosystems, USA). Primer Express 3.0 Software (Applied Biosystems, SA) were used to design and analyze primers for PCR reaction. The oligonucleotide primer sequences for qPCR are listed in Table 1. Thermal cycle of PCR consisted of an initial denaturation at 95 °C for 10 min., followed by 40 cycles each of 95 °C for 15 seconds and 60 °C for 1 min. each. A melting curve analysis was performed after the PCR. The cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ approach was used to investigate the relative quantification of distinct mRNA levels (42). All reactions were carried out 3 times in duplicate using 3 different samples. The relative expression levels were normalized to amplification of *α-tubulin* (*Os03g0726100*) gene. Statistical analysis to assess the significant difference of relative expression of particular genes among nontreatment and different treatment was performed using SPSS version 20.0 (SPSS, Inc., USA) for one-way analysis of variance followed by Duncan's Multiple Range Test. Means with the different letters are significantly different at $p < 0.05$ level.

Results

Effect of NaCl stress on rice seedling growth

The 3 rice cultivars with differing salt stress tolerance (VD20, OM6976 and AGPPS114) were examined for growth parameters after a dose-response experiment. All rice cultivars experienced significantly reduced shoot and root growth as a result of salt stress. There were no visible signs at the beginning of salt treatment, but the decrease in shoot and root growth occurred as exposure salt concentration increased (Fig. 1A). The root growth of VD20, OM6976, and AGPPS114 cultivar seedlings decreased significantly with an increase in salt concentration from 100 mM, 150 mM and 250 mM respectively (Fig. 1B). The root length of all three cultivars was shorter, over 50 %, in comparison to their respective controls after 3 days of treatment at these NaCl concentrations. The root growth of VD20, OM6976 and AGPPS114 seedlings were almost inhibited at high NaCl concentration (from about 150 mM, 200 mM and 300 mM to 500 mM respectively).

The effect of salt stress to shoot growth depended on the cultivar. From 50 mM of NaCl concentration treatment, a gradual decrease was found in shoot length of VD20 cultivar during increasing NaCl concentration; however, there was no significant effect of salt treatment on AG-



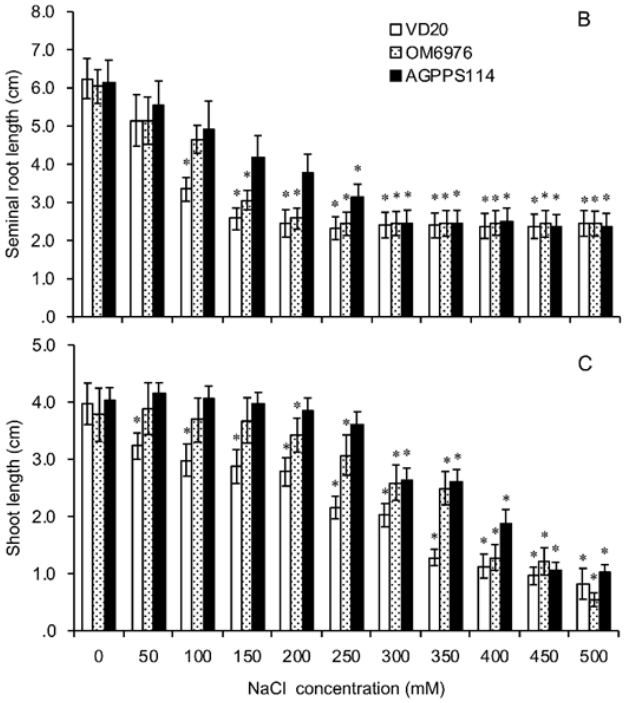


Fig. 1. Effect of salt treatment on the growth of rice seedlings. (**A**) Morphological of rice seedlings under salt treatment (A). Root and shoot length in control and salt treatment (**B** and **C**). Six day old rice seedlings were treated with different concentrations of NaCl for 3 d, and root and shoot lengths were measured. Data are mean ± SD. *Significant mean difference from control at p≤0.05 was determined with multiple comparisons by Tukey test. White bar, 2 cm

PPS114 until 300 mM of NaCl concentration (Fig. 1C). OM6976 cultivar showed a significantly decreased shoot length from 200 mM of NaCl treatment compared to the control shoot length.

Na+ accumulation and Na+ localization in root and shoot seedlings under salt stress

Na^+ concentration was significantly increased in the rice roots and shoots in all cultivars with increasing NaCl concentration treatments (Fig. 2A). NaCl concentration (200 mM) induced sodium ion accumulation in plant tissue, with

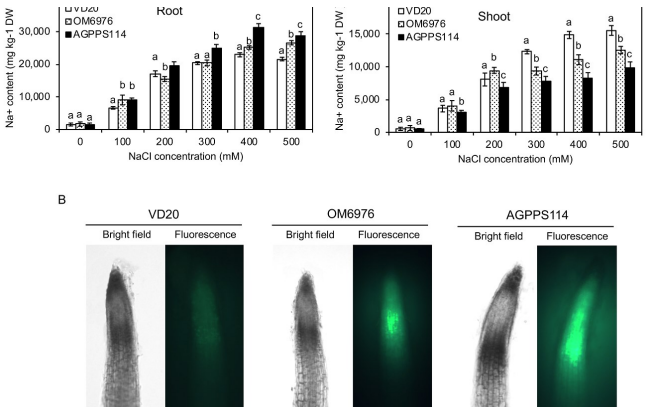


Fig. 2. Changes in sodium (Na^+) content in the roots and shoots of rice seedlings subjected to NaCl stress for 3 days (A). Data presented are mean ± SD. Different letters indicate a significant difference with multiple comparisons by Tukey test at p≤0.05. Na^+ localization traced by CoroNa Green in the rice roots after being treated with 200 mM NaCl, green represents Na^+ (B). White bar, 200 mm.

over 11.2-fold higher Na^+ content in VD20, 9.3-fold more Na^+ in OM6976 and 13.5-fold more Na^+ in AGPPS114 against their respective controls. The sodium ion concentration of root seedlings of the tolerant cultivar (AGPPS114) was significantly higher than that of the sensitive cultivar (VD20). There was no significant difference in sodium concentration between VD20 cultivar and OM6976 cultivar. On the contrary, the accumulation of sodium ions in the leaf tissues of salt sensitive rice VD20 cultivar was greater than OM6976 and AGPPS114 cultivar. Sodium ion accumulations in root tissues of the cultivars after treatment with 200 mM NaCl were confirmed by CoroNa Green staining (Fig. 2B). The green fluorescence of Na^+-CoroNa Green in salt stressed roots of OM6976 and AGPPS114 cultivar was clearly detected, especially at the root elongation zone.

Salt stress-induced reactive oxygen species (ROS) generation and lipid peroxidation in root seedlings

To investigate whether salt stress induced ROS production, the seedlings were treated with NaCl for 24 hrs and the production of ROS in the root seedlings was analyzed histochemically. The formation of more intensity of the blue colored pigment formazan under NBT treatment indicated higher $O_2^{\cdot-}$ production in the root seedlings of the salt-sensitive rice cultivar compared to the tolerant cultivar roots. In addition, elevated H_2O_2 production was indicated in the roots of the sensitive cultivar due to the higher brown polymerization product generated from the interaction of H_2O_2 with DAB. The ROS production increased gradually in VD20 roots with the increasing salt concentration; however, there was no significant change of ROS production in OM6976 and AGPPS114 until 200 and 300 mM of salt concentration treatment respectively (Fig. 3). Furthermore, salt stress significantly impacted ROS-induced lipid peroxidation in rice roots. Under salt treatment, Schiff's reagent staining was found in the VD20 cultivar root tips (Fig. 4). However, during salt stress, the lipid peroxidation level of OM6976 and AGPPS114 roots increased slightly.

Analysis of antioxidant enzyme activity

Significant differences in specific activity of the antioxidant enzymes exhibited in the roots of VD20, OM6976 and AGPPS114 cultivars. In the roots of salt-tolerant cultivars, the overall specific activity of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), was increased. After 6, 12 and 24 hrs of salt treatment, the SOD activity, which converts $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2), was considerably higher in OM6976 and AGPPS114 roots than in VD20 roots (Fig. 5A). In addition, the specific CAT activity was more significant in roots of the salt -tolerant cultivars (OM6976 and AGPPS114) in comparison with the salt-sensitive cultivar (VD20) at 12 and 24 hr of salt treatment (Fig. 5A). The in-gel antioxidant enzyme activity assay correlated with total antioxidant enzyme activity (Fig. 5B). In-gel SOD assay indicated a higher-level expression of SOD activity in OM6976 and AGPPS114 cultivar roots compared to VD20 roots. Furthermore, an isozyme of CAT appeared from 12 hrs in OM6976 roots and from six hours in AGPPS114 of time-course analysis.

Effect of salt stress on proline and glycine betaine accumulation

The proline concentration in the salt-tolerant cultivar increased as the salt levels increased. However, proline accumulation in the salt-sensitive (VD20) and moderately

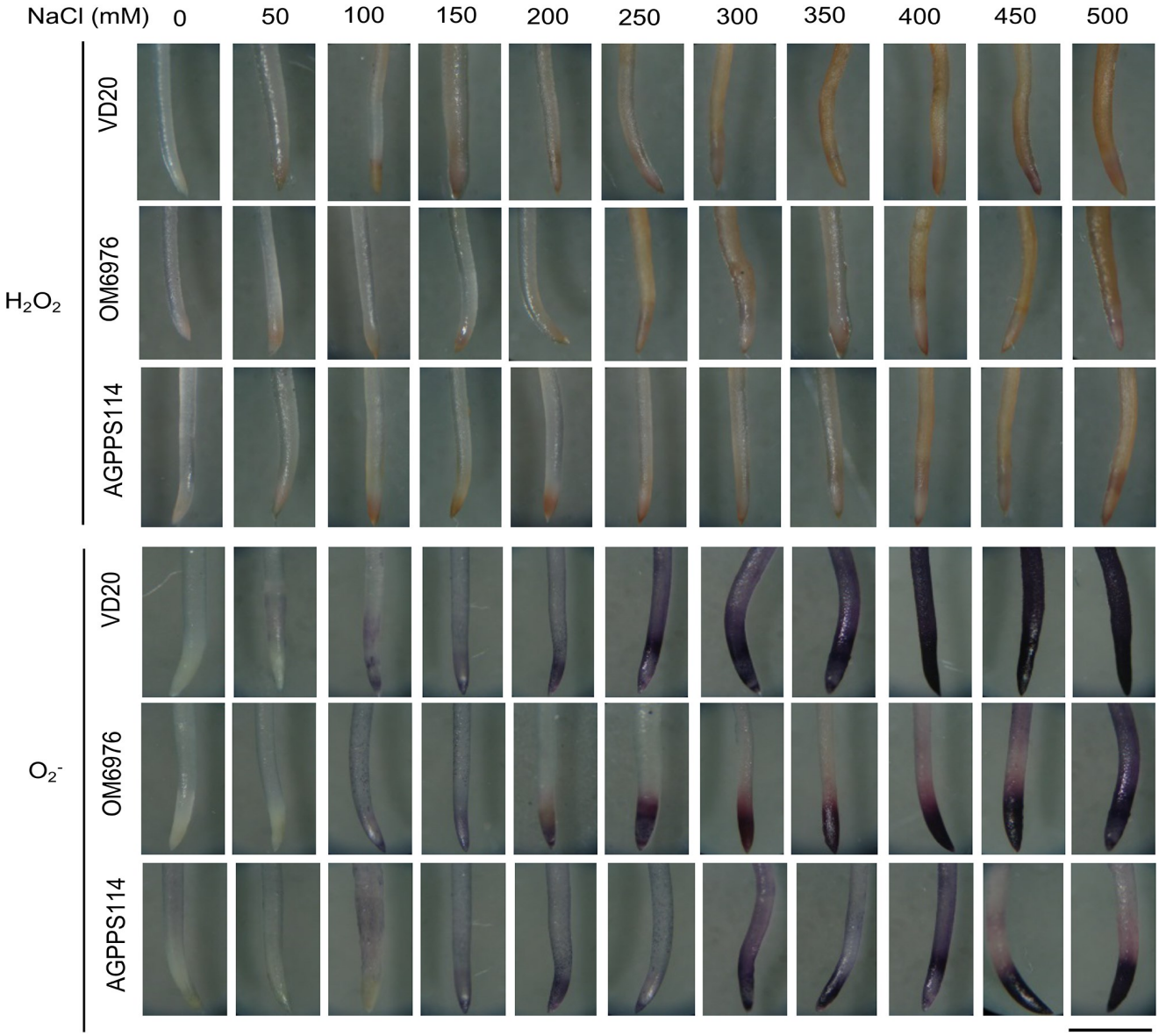


Fig. 3. Histochemical detection of ROS production and lipid peroxidation in the rice roots under salt stress. H_2O_2 and O_2^- detection through DAB and NBT staining, respectively, at varying NaCl concentrations of 6 days old rice seedlings. The intensity of stains represents the degree of sensitivity in NaCl stress. Black bar, 0.5 cm.

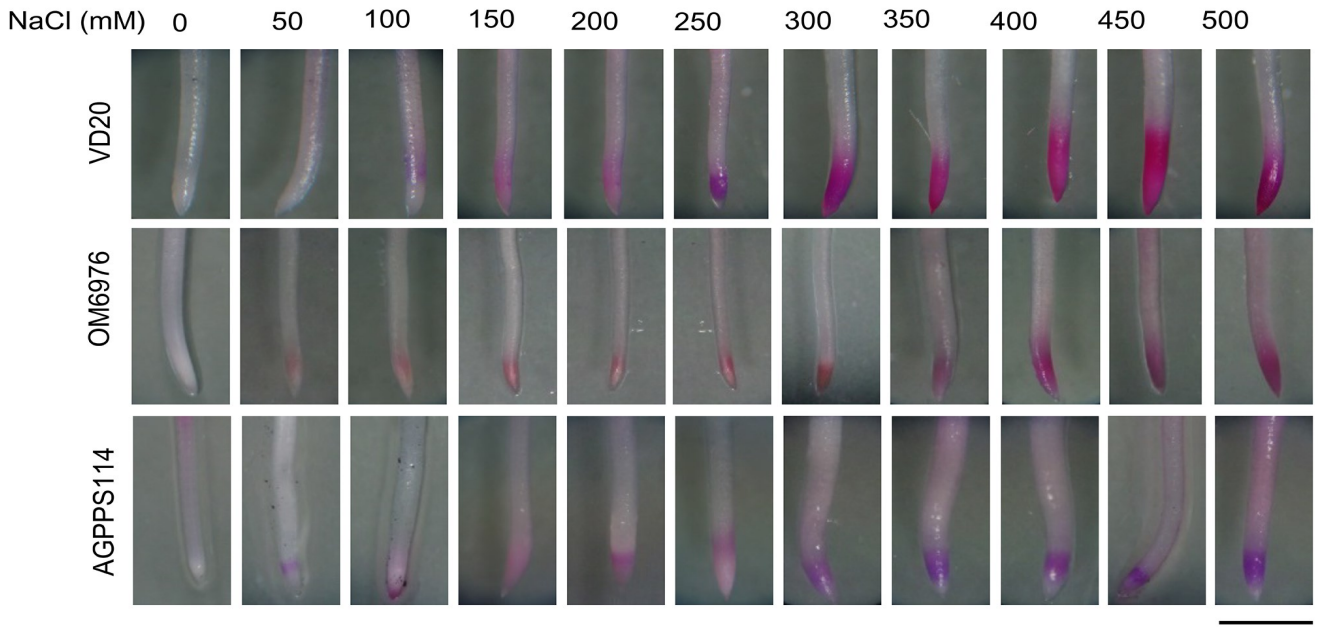


Fig. 4. Histochemical detection of lipid peroxidation in the rice roots under salt stress. Peroxide generation by ROS detected by Schiff's reagent at varying NaCl concentrations of 6 days old rice seedlings. Black bar, 0.5 cm.

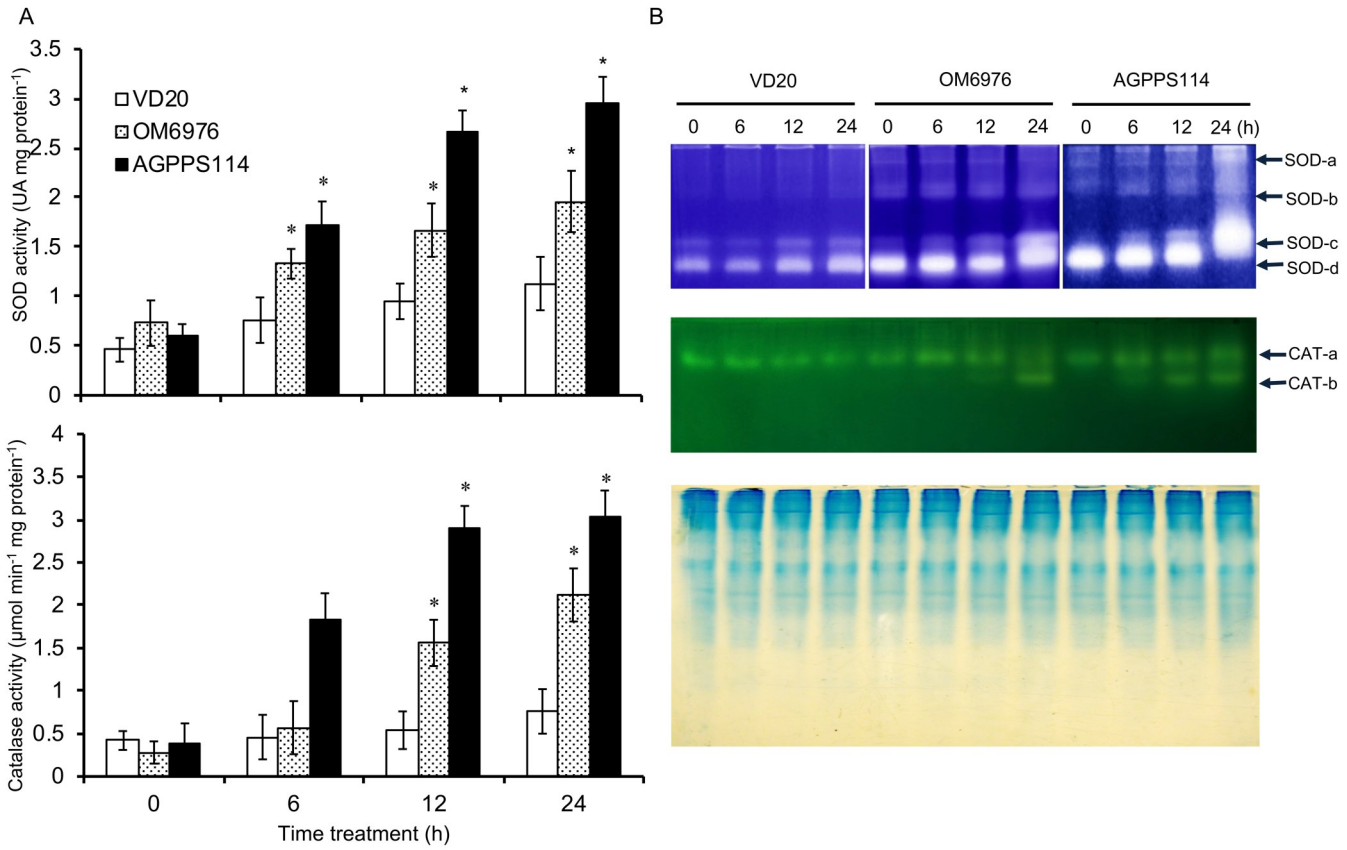


Fig. 5. Activity detection of antioxidant enzyme in the rice roots under salt stress. Activity of SOD and CAT *in vitro* (**A**) and polypeptide polymorphism of SOD and catalase through in gel staining (**B**) of 6 days old rice seedling under varying NaCl concentrations. Isozyme analysis of SOD showing the existence of four bands corresponding to SOD isoforms with variable intensities in the control and the stressed rice seedlings. Data presented herein are the means of three replication and significantly different from the control at p≤0.05 by paired *t* test (*). Equal amounts of protein on each time point were assessed by Coomassie blue staining.

tolerant cultivar (OM6976) decreased significantly at 150 mM of NaCl concentration treatment (Fig. 6A). Over 150 mM of NaCl, the highest proline content was only found in AGPPS114. AGPPS114 accumulated approximately 7.7-fold higher proline content while OM6976 and VD20 accumulated 3.0- and 2.1-fold higher proline content respectively, as compared to the controls (Fig. 6A).

The glycine betaine production increased in all the cultivars exposed to salinity stress. No significant difference was observed in glycine betaine concentration among the rice cultivars when NaCl concentration treatment was 50 mM (Fig. 6B). However, glycine betaine levels were reduced in roots of VD20, OM6976, and AGPPS114 at 100 mM, 150 mM and 250 mM respectively, of salt concentration treatment. In comparison to the control, AGPPS114 accumulated approximately 1.9-fold higher glycine betaine while OM6976 and VD20 accumulated 1.5- and 1.3-fold higher glycine betaine content respectively (Fig. 6B).

Gene expression in rice roots under salt stress

In order to determine whether salinity stress induced the change in gene expression profiles in rice roots, the transcription level of 4 Na^+/K^+ transport regulation-related genes was analyzed for the 3 cultivars. The time-course experiment indicated induction of the genes related to the transport occurs very rapidly under salt stress in the cultivars.

The expression analysis of *OsHKT1* of *HKTs* (*High-Affinity Potassium Transporters*) family genes showed higher expression in AGPPS114 and OM6976 than VD20 under salt stresses (Fig. 7A, 7B). *OsHKT1;4* and *OsHKT1;5* were rapidly decreased in VD20 after 6 hrs of salt treatment (Fig. 7B). Likewise, the expression level of *OsNHX1* (*Oryza sativa* Na^+/H^+ *antiporters1*) was significantly increased under salt treatment in both salt-tolerant cultivars (Fig. 7A). *OsNHX1* reached the highest level of transcripts 12 hrs under salt stress in OM6976 and AGPPS114, which increased over 19 and over 27-fold, respectively (Fig. 7B). On the other hand, the expression of this gene decreased 3-fold after salt treatment 12 hrs in VD20. The expression of *OsSOS1* (*Oryza sativa Salt Overly Sensitive1)* significantly changed in AGPPS114 (Fig. 7B). The maximum *OsSOS1* expression was achieved 24 hrs of salt treatment and was over 21-fold (Fig. 7A, 7B). In VD20, *OsSOS1* expression decreased over 2-fold after 6 hrs. The expression of *OsSOS1* gene did not change significantly in OM6976 under salinity stress.

Discussion

Rice is the most widely cultivated food crop worldwide but is considered a salt-sensitive crop. Salinity stress is one of the major limiting factors in rice yield (43-45). The seedling was indicated as a critical stage to determine successful crop production (46). According to one study (47), osmotic stress by salinity was the primary factor that reduced seedling growth. It was also reported that a reduction of the shoot-root length of rice cultivars under NaCl stress (48). In this study, salinity significantly affects the seedling height of different rice cultivars. Similar rice and other crops have been reported (49, 50). Increasing salt concentration leads

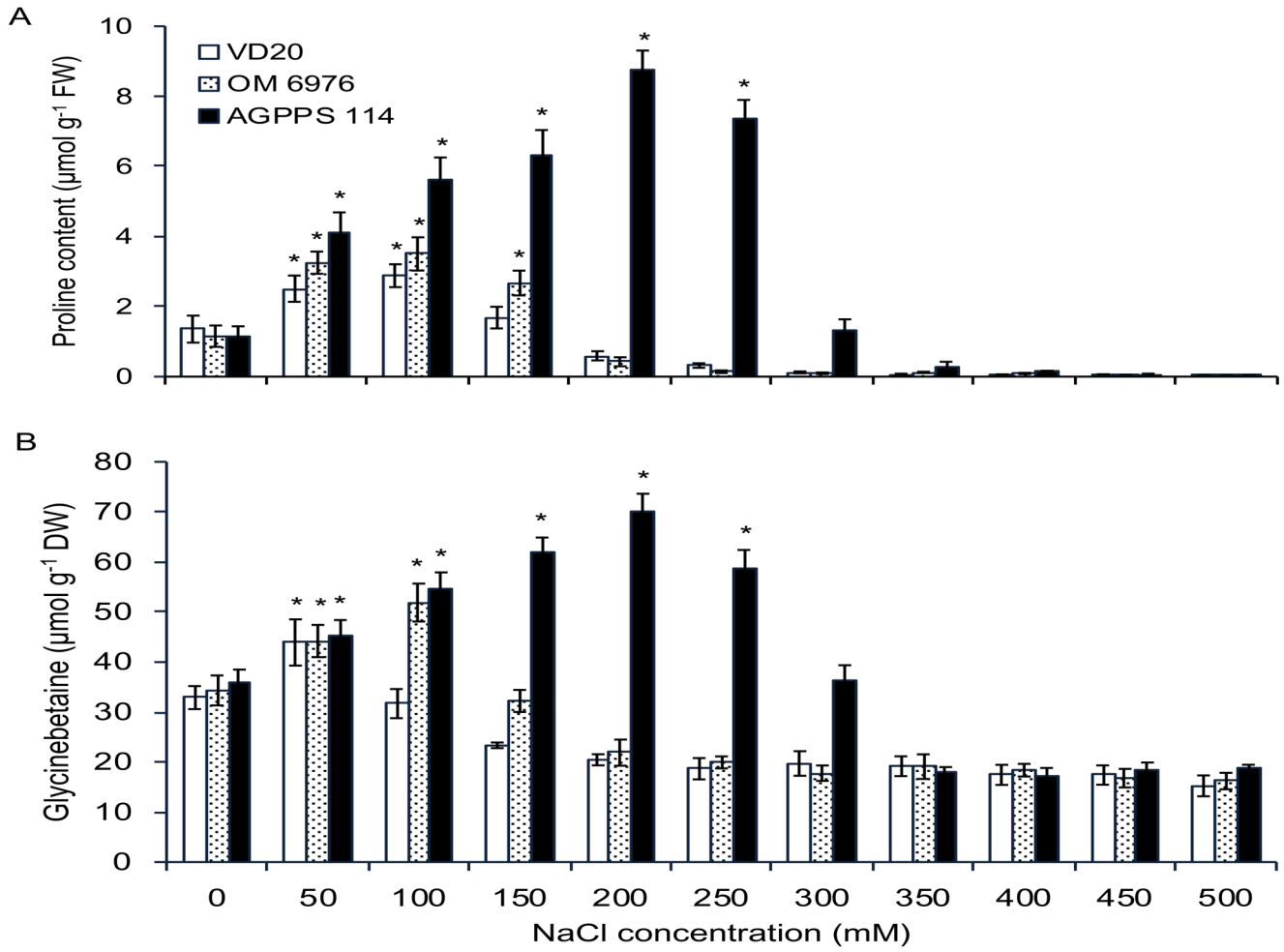


Fig. 6. Accumulation of proline (**A**) and glycinebetaine (**B**) in control and the salt-stressed seedlings. The data are mean ± SD. *Significantly different from the control at p≤0.05 by paired *t* test.

to a reduction in shoot/root length but different cultivars levels, so it is essential to investigate the differences in salt stress tolerance and transport control mechanism among the cultivars under salt stress.

The antioxidant enzyme system in root tissues can guard against ROS and play a role in various physiological processes in plant roots, including stress tolerance, oxidative damage and apoptosis induction (51). Increased plant antioxidant response strongly correlates with reduced oxidative damage and enhanced salinity tolerance (16, 52-56). The ROS-scavenging antioxidant enzyme activity was analyzed to determine the correlation between antioxidant response and salinity tolerance. The antioxidant enzyme activity in the rice cultivars differed significantly in response to salt treatment. The present data indicated that salt-tolerant cultivars (AGPPS114 and OM6976) differed obviously from salt-sensitive cultivar (VD20) in the activities of the antioxidant enzymes under salt stress, with SOD and CAT activities for the AGPPS114 and OM6976 roots increased rapidly in comparison to VD20. Previous studies indicated that increased salt concentration reduces the antioxidant enzyme activities, including CAT in the salt-sensitive cultivar rice, but enhances CAT activities in salt-tolerant cultivar rice (53, 56, 57). In this study, in comparison to the control, the CAT activities decreased with the increasing of salt treatment in salt-sensitive cultivar (VD20).

Proline, as well as glycine betaine, usually accumulate at high concentrations in plants under salt stress (15). Proline and glycine betaine can prevent cells from damage by acting as an osmotic agent and reducing the oxidative damages caused by ROS during salinity stress (54, 58). In the present study, the proline and glycine betaine accumulation in AGPPS114 and OM6976 cultivar during salt treatment was significantly higher than salt-sensitive cultivar VD20 at mild salt stress concentrations. Compared to the control, among the salt-tolerant rice cultivars, AGPPS114 accumulated 4.9-fold higher proline content while the other cultivar OM6976 accumulated 3.0-fold higher proline content at 100 mM NaCl. Similarly, glycine betaine content increased about 1.5-fold higher in AGPPS114 and OM6976 at 100 mM NaCl than in control. The proline production was also higher at 50 and 100 mM NaCl in VD20; however, the production reduced at higher concentrations of NaCl treatment (over 100 mM NaCl). OM6976 showed similar responses to that of VD20. The reduction in proline and glycine betaine production in the salt-sensitive rice cultivar and also in salt-tolerant rice cultivars at high levels of salinity was found, most likely due to decreased proline and glycine betaine production or increased breakdown of these amino acids under high salt stress. In previous reports, a positive association between

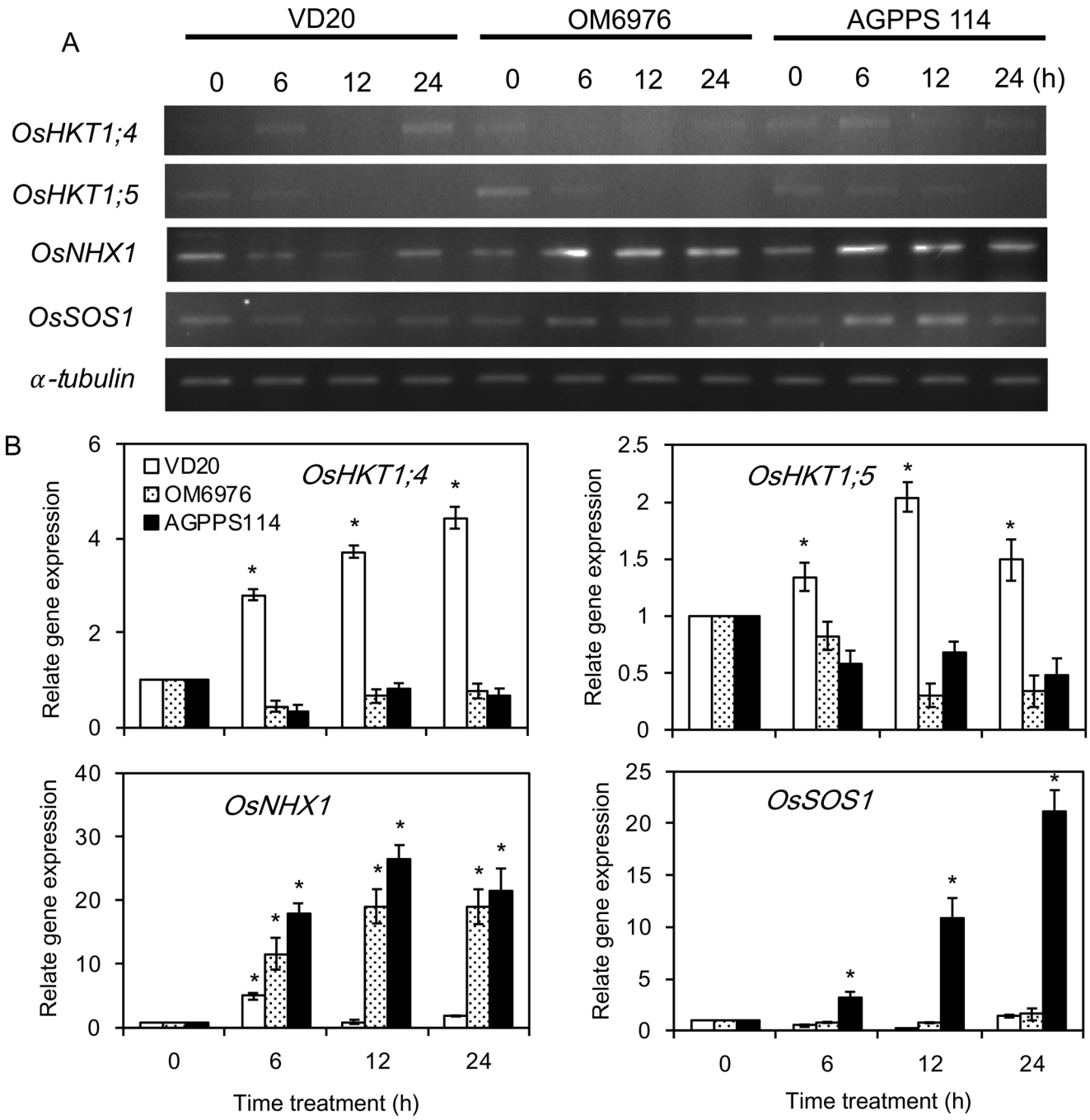


Fig. 7. Expression of the Na^+/K^+ transport regulation-related genes by RT-PCR (**A**) and quantitative real-time PCR (**B**) in the rice roots under salt treatment. *Significantly different from the control at p≤0.05 by paired *t* test. *α-tubulin* (*Os03g0726100*) gene was used as a reference gene.

the production of proline, glycine betaine and stress tolerance has been demonstrated in plants (59-61). Higher proline and glycine betaine content in AGPPS114 might lead to higher salinity stress tolerance than the other cultivars. It was also investigated that increased salt stress tolerance is related to the enhanced capacity of the antioxidant system in rice (53). Because proline and glycine betaine scavenge free radicals and reduce ROS generation, they have been suggested to play significant roles as antioxidants in protecting cells against stresses.

The plasma membrane-localized salt overly sensitive (SOS1) and the vacuole membrane (tonoplast) localized NHX1 are two Na^+/H^+ antiporters that played essential roles in Na^+ efflux back to the soil and compartmentalization of Na^+ in the vacuole, respectively (62-65). Na^+ transportation into the vacuole via NHX1 is one of the most critical salt defense mechanisms under salinity stress in rice. *OsNHX1* gene was found up-regulation in the Pokkali cultivar (salt-tolerant) roots exposed to 200 mM NaCl (66). In previous studies, the transcript level of different isoforms of the rice *OsNHX* gene, including *AtNHX1, 2* and *5* in *Arabidopsis thaliana* (67) and *PeNHX1*, *2*, *3*, *5* and *6* in *Populus euphratica* (68) was induced under salt stress. The present study indicated the significant increase of *OsNHX1* and *OsSOS1* gene expression under salt stress in AGPPS114 cultivar (salt-tolerant rice) and non-significant up-regulation in VD20 cultivar (salt-sensitive rice). The high transcript levels of *OsNHX1* under salt stress can cause high activities of tonoplast Na^+/H^+ antiporter in AGPPS114 and OM6976 cultivars. Accumulation of Na^+ and transcript level of *OsNHX1* in AGPPS114 and OM6976 roots might be correlated with each

other. The increase of *OsNHX1* transcript level might reduce sodium ions transfer from root to shoot by accumulating sodium ions in vacuoles. It was found that acidification of the vacuolar lumen induces Na^+/H^+ antiporters and its alkalization may adjust the *H^+ pump* gene expression (69). Overexpression of *H^+ pumps* in correlation to Na^+/H^+ antiporter may control plant salinity tolerance mechanisms. The previous study indicated that the transcript level of SOS1 is up-regulated by high salinity (70). In the present study, transcript levels of *SOS1* in the AGPPS114 cultivar were significantly induced during salt stress. As a result, a high abundance of *OsSOS1* gene expression in the AGPPS114 may contribute to reduced accumulation of Na^+ in the AGPPS114 under salt stress conditions.

Members of the HKT1 family of HKTs are related to the regulation of long-distance transport of Na^+ through its reabsorption from the xylem sap into the parenchyma cells of the root, preventing the accumulation at a high level of sodium ions in the shoot (71, 72). Induced expression of *OsHKT1* in rice under salt stress was also reported (73, 74). In the condition of K^+ deficiency, OsHKT1 specifically induced Na^+ uptake in rice roots (75). Under salinity stress conditions, Na^+ competes at K^+ binding sites and excess Na^+ entering the cytosol increases Na^+/K^+ ratio in the cytosol, resulting in K^+ deficiency (76, 77). OsHKT1;5 was played an essential role in the exclusion of Na^+ from leaves by transporting Na^+ from xylem sap to roots (78). In addition, OsHKT1;5 functions by transferring Na^+ from the xylem vessel to the xylem parenchyma to minimize the negative effects of Na^+ accumulation on the plant (79). The higher uptake of Na^+ into the cytosol of the salt-tolerant cultivars (AGPPS114 and OS6976) may lead to higher expression of *OsHKT1* than in the salt-sensitive cultivar (VD20) as reported earlier (80).

Conclusion

In conclusion, salinity stress caused significant physiological changes in the root of the rice cultivar seedlings, including increased Na^+content, coupled with a higher concentration of ROS and more lipid peroxidation. The more tolerance of the AGPPS114 and the OM6976 seemed to be associated with more capability to exclude Na^+ via ion transporter regulation and higher activity of antioxidant enzymes during salinity stress. The present results also indicated that high levels of proline and glycine betaine accumulation in the AGPPS114 might confer salt tolerance specifically at the seedling stage. Interestingly, OM6976 cultivar, which was previously indicated as moderately tolerant, showed similar responses to the salt-sensitive cultivar by less proline and glycine betaine accumulation at high salt stress concentration. The salt-tolerant cultivar maintained higher antioxidant defense and ion transporter systems with elevated proline and glycine betaine accumulation levels than salt-sensitive rice during salinity stress at the seedling stage. As a result, the findings of this study will aid in the examination of essential elements that cause variances in salt tolerance among rice cultivars and will serve as a guide for the growth of salt-tolerant rice types.

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Authors contributions

TNN and NTP designed research and performed experiments. TNN and LHT analyzed experiment data. TNN and DQC wrote the manuscript paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors do not have any conflict of interests to declare.

Ethical issues: None

Supplementary data

Supplementary Table : Salt tolerance of 12 rice cultivars under salt stress (6‰ NaCl) after three weeks.

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